PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 19 October 2000 (19.10.00)	
International application No. PCT/SG98/00103	**Applicant's or agent's file reference** GM/AY/R33-59
International filing date (day/month/year) 11 December 1998 (11.12.98)	**Priority date** (day/month/year)
Applicant FANG, Rong-Xiang et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

06 July 2000 (06.07.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Olivia TEFY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rul s 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
GM/AY/RN/R33-59		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/SG 98/00103	11/12/1998	
Applicant		
INSTITUTION OF MOLECULAR AGROBIOLOGY et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [X] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 11

   [ ] as suggested by the applicant.

   [X] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/SG 98/00103

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/62 C07K14/00 A01H5/00 C07K14/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 773 705 A (CALLIS JUDY ET AL) 30 June 1998 (1998-06-30) the whole document ---	1-3,5, 10-13, 19,25, 26,28-30
X	WO 90 02189 A (UPJOHN CO) 8 March 1990 (1990-03-08) the whole document ---	6-9, 15-18, 20,23, 31,32
X	EP 0 672 754 A (KANEBO LTD) 20 September 1995 (1995-09-20) page 1 -page 5; claims 1-12,17,18; examples 1-5 --- -/--	15-17, 20,31,32

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 November 1999	29. 11. 99

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Oderwald, H

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/SG 98/00103

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 21018 A (ASGROW SEED CO ;BOESHORE MAURY L (US); MCMASTER J RUSSELL (US); TR) 11 July 1996 (1996-07-11) claims 12-22,38-45; figures 1-5,8; example 1	23

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/SG 98/00103

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5773705 A	30-06-1998	NONE	
WO 9002189 A	08-03-1990	AT 105586 T	15-05-1994
		AT 160173 T	15-11-1997
		AU 639891 B	12-08-1993
		AU 3970489 A	23-03-1990
		AU 634168 B	18-02-1993
		AU 3987089 A	23-03-1990
		CA 1332718 A	25-10-1994
		CA 1329561 A	17-05-1994
		CN 1044126 A	25-07-1990
		CN 1044297 A	01-08-1990
		DE 68915282 D	16-06-1994
		DE 68915282 T	29-09-1994
		DE 68928445 D	18-12-1997
		DE 68928445 T	20-05-1998
		DK 28191 A	19-02-1991
		EP 0429478 A	05-06-1991
		EP 0429483 A	05-06-1991
		EP 0693555 A	24-01-1996
		EP 0699757 A	06-03-1996
		JP 4500151 T	16-01-1992
		JP 4500152 T	16-01-1992
		WO 9002184 A	08-03-1990
EP 0672754 A	20-09-1995	JP 2880024 B	05-04-1999
		JP 5328977 A	14-12-1993
		JP 6169789 A	21-06-1994
		US 5618699 A	08-04-1997
		WO 9320217 A	14-10-1993
WO 9621018 A	11-07-1996	AU 706875 B	24-06-1999
		AU 2768795 A	24-07-1996
		EP 0871739 A	21-10-1998
		TR 960651 A	21-07-1996

INTERNATIONAL SEARCH REPORT

In. ational application No.
PCT/SG 98/00103

Box I Obs rvation wh re ertain laims wer found unsearchable (Continuation f item 1 f first sh t)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-5, 10-14, 19, 21, 22, 25, 26, 28-30

   A method for enhancing the protein production in a plant cell or a plant utilizing a ubiquitin fusion protein, said fusion protein, a vector encoding said fusion protein, a plant cell or a plant comprising said vector, a nucleic acid comprising or consisting of SEQ ID NO: 1, a protein comprising or consisting of SEQ ID NO: 2.

2. Claims: 6-9, 15-18, 20, 23, 24, 27, 31, 32

   A method for enhancing the protein production in a plant cell or a plant utilizing a fusion protein comprising a peptide from the cucumber mosaic virus coat protein, said fusion protein, a vector encoding said fusion protein, a plant cell or a plant comprising said vector, a nucleic acid comprising or consisting of SEQ ID NO: 3, a protein consisting of SEQ ID NO: 4.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-5, 10-14, 19, 21, 22, 25, 26, 28-30

   A method for enhancing the protein production in a plant cell or a plant utilizing a ubiquitin fusion protein, said fusion protein, a vector encoding said fusion protein, a plant cell or a plant comprising said vector, a nucleic acid comprising or consisting of SEQ ID NO: 1, a protein comprising or consisting of SEQ ID NO: 2.

2. Claims: 6-9, 15-18, 20, 23, 24, 27, 31, 32

   A method for enhancing the protein production in a plant cell or a plant utilizing a fusion protein comprising a peptide from the cucumber mosaic virus coat protein, said fusion protein, a vector encoding said fusion protein, a plant cell or a plant comprising said vector, a nucleic acid comprising or consisting of SEQ ID NO: 3, a protein consisting of SEQ ID NO: 4.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference GM/MC/R33-59	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416).
International Application No. **PCT/SG98/00103**	International Filing Date *(day/month/year)* 11 December 1998	Priority Date *(day/month/year)* 11 December 1998
International Patent Classification (IPC) or national classification and IPC **Int. Cl.** [7] C12N 15/82, 15/62 C07K 14/00 A01H 5/00 C07K 14/08		
Applicant INSTITUTE OF MOLECULAR AGROBIOLOGY et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheet(s).

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☒	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☐	Certain observations on the international application

Date of submission of the demand	Date of completion of the report
6 July 2000	2 February 2001
Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized Officer **TERRY MOORE** Telephone No. (02) 6283 2632

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed.

[ ] the description, pages , as originally filed,
pages , filed with the demand,
pages , received on with the letter of

[ ] the claims, pages , as originally filed,
pages , as amended (together with any statement) under Article 19,
pages , filed with the demand,
pages , received on with the letter of

[ ] the drawings, pages , as originally filed,
pages , filed with the demand,
pages , received on with the letter of

[ ] the sequence listing part of the description:
pages , as originally filed
pages , filed with the demand
pages , received on with the letter of

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, was on the basis of the sequence listing:

[X] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

4. [ ] The amendments have resulted in the cancellation of:

[ ] the description, pages

[ ] the claims, Nos.

[ ] the drawings, sheets/fig.

5. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report*

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:

The Authority found that there were two inventions claimed.

The first invention corresponds to claims 1-5, 10-14, 19, 21, 22, 25, 26 and 28-30. This invention resides in a fusion construct comprised of an N-terminal ubiquitin monomer fused to a C-terminal protein of interest, wherein expression of the fusion construct is driven by a promoter other than the ubiquitin promoter.

The second invention corresponds to claims 6-9, 15-18, 20, 23, 24, 27, 31 and 32. This invention resides in a fusion construct comprising an N-terminal segment of the cucumber mosaic virus coat protein gene fused to a C-terminal protein of interest.

Although both inventions provide enhanced expression of fusion peptides in plant expression systems, this is not novel. It is well known in the art that N-terminal fusion peptides can provide enhanced expression. As such this feature does constitute a novel unifying feature and the two invention lack unity *a posteriori*.

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	**4, 6-9, 14, 21, 22, 24, 27**	**YES**
	Claims	**1-3, 5, 10-13, 15-20, 23, 25, 26, 28-32**	**NO**
Inventive step (IS)	Claims	**6-9, 14, 27**	**YES**
	Claims	**1-5, 10-13, 15-26, 28-32**	**NO**
Industrial applicability (IA)	Claims	**1-32**	**YES**
	Claims		**NO**

2. Citations and explanations (Rule 70.7)

The following documents identified in the International Search Report have been considered for the purposes of this report:

D1 US 5 773 705 (Vierstra, RD et al) 30 June 1998

D2 WO 90 02189 (THE UPJOHN CO) 8 March 1990

D3 EP 0 672 754 (KANEBO LTD) 20 September 1995

D4 WO 96 21018 (ASGROW SEED CO) 11 July 1996

New Citations

D5 GENPEPT ACCESSION NO: AAC49970 18 March 1998 Karrer et al

D6 GENBANK ACCESSION NO: X89652 25 March 1997 Haq et al

Novelty and Inventive Step

The invention described in the specification comprises two kinds of N-terminal fusion constructs, each providing enhanced expression in plant systems. The first construct involves comprises fusion of a ubiquitin monomer with a peptide of interest wherein the promoter driving expression of the fusion construct is not the native ubiquitin promoter. The second construct involves the fusion of the N-terminal region of the cucumber mosaic virus coat protein NP14 with a protein of interest.

D1 describes fusions in which a ubiquitin monomer is fused to the N-terminus of a protein of interest and expression of the fusion construct is driven by the CaMV 35S promoter. The disclosed constructs provide enhanced levels of expression in plant expression systems. As such the citation discloses the general principle of enhanced expression using ubiquitin monomers and recognises that elevated levels of expression are not dependent on the use of the ubiquitin promoter. It also discloses the amino acid sequence described as SEQ ID NO: 2 and a DNA sequence equivalent to SEQ ID NO 1. In light of the information disclosed in D1 claims 1-3, 5, 10-13, 19, 25, 26 and 28-30 lack novelty and claims 4, 14, 21 and 22 an inventive step.

D2 describes use of the 5' untranslated sequence from the cucumber mosaic virus coat protein to provide a chimeric vector for the expression of foreign proteins in plant cells. However this does not disclose or teach toward the fusion of translated coat protein sequence with a peptide of interest. As such the citation does not impinge on the novelty or inventive merit of the claims.

Continued in supplemental box.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of BOX V2

D3 discloses fusion of cucomovirus coat protein coding sequence with a peptide to provide a mechanism for expressing a gene of interest in plant cells. However the citation does not suggest use of only the N-terminal region of the coat protein or indicate that this region has special properties with regard to enhanced expression or stability of recombinant fusion peptides. Therefore although the citation discloses fusion constructs comprising regions of the coat protein that include SEQ ID NO: 4, thereby depriving claims 15-18, 20, 23, 31 and 32 of novelty, the citation does not disclose the specific sequence of SEQ ID NO: 4 or its unique properties.

D4 discloses the coat protein sequence of the cucumber mosaic virus. However it does not disclose any specific properties for the N-terminal region of the protein, or suggest the use of the protein to provide enhanced expression of fusion peptides. Therefore, although the citation discloses a nucleic acid comprising SEQ ID NO: 3 and thus deprives claims 23 of novelty, it does not deprive any further claims of either novelty or inventive step.

D5 discloses the nucleic acid and amino acid sequence of the tobacco ubiquitin monomer. As such it discloses the exact sequences defined in claims 25 and 26 and sequences equivalent to those defined in claims 21 and 22. Therefore claims 25 and 26 lack novelty and claims 21 and 22 an inventive step in light of D5.

D6 discloses the nucleic acid and amino acid sequence of the cucumber mosaic virus coat protein. As such it discloses a sequence containing the sequence defined in claim 23 and deprives the claim of novelty.

Industrial Applicability

Claims 1-32 define methods, vectors, plants and peptides useful in the are of agricultural molecular biology.

----------**NEW CITATIONS**----------

GENPEPT ACCESSION NO: AAC49970 18 March 1998 Karrer et al

GENBANK ACCESSION NO: X89652 25 March 1997 Haq et al

VERSION*
PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :		(11) International Publication Number:	WO 00/36129
C12N 15/82, 15/62, C07K 14/00, A01H 5/00, C07K 14/08	A1	(43) International Publication Date:	22 June 2000 (22.06.00)

(21) International Application Number: PCT/SG98/00103

(22) International Filing Date: 11 December 1998 (11.12.98)

(71) Applicant *(for all designated States except US)*: INSTITUTE OF MOLECULAR AGROBIOLOGY [SG/SG]; 1 Research Link, Singapore 117604 (SG).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: FANG, Rong-Xiang [CN/CN]; Institute of Microbiology, Zhong Guan Cun, Beijing 100080 (CN). WU, Jun-Lin [CN/CN]; Institute of Microbiology, Zhong Guan Cun, Beijing 100080 (CN). CHEN, Xiao-Ying [CN/CN]; Institute of Microbiology, Zhong Guan Cun, Beijing 100080 (CN).

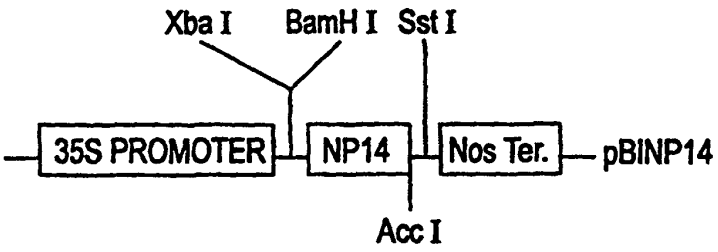
(74) Agent: ELLA CHEONG & G. MIRANDAH; P.O. Box 0931, Raffles City, Singapore 911732 (SG).

(81) Designated States: CN, JP, SG, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

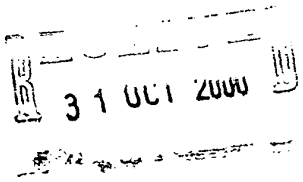
Published
With international search report.

(54) Title: ENHANCED PROTEIN PRODUCTION IN HIGHER PLANTS BY N-TERMINAL FUSION OF A UBIQUITIN OR A CUCUMBER MOSAIC VIRUS COAT PROTEIN PEPTIDE

(57) Abstract



Methods are disclosed for enhancing protein production. One method comprises preparing a vector by inserting a gene encoding ubiquitin in front of a gene encoding a protein of interest and inserting the vector into a cell. A fusion protein will be expressed which includes ubiquitin plus the protein of interest. Ubiquitin C-terminal hydrolases can cleave the fusion protein leaving the desired protein in its free state. This method causes enhanced production of the protein of interest as compared to performing the same method without the ubiquitin gene as part of the vector. A ubiquitin promoter is unnecessary to yield this enhanced production and is not used. A second method is very similar except that in place of a ubiquitin gene, a gene encoding fourteen amino acids of cucumber mosaic virus coat protein is inserted in front of the gene of interest. This results in expression of a fusion protein comprising the fourteen amino acid residues of the coat protein bonded to the protein of interest. The fusion protein is produced at a higher level than is the protein when the coat protein gene fragment is not present in the vector. In both methods the genes can be placed under the control of heterologous promoters such as a 35S promoter.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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EE	Estonia	**LR**	Liberia	**SG**	Singapore		

TITLE OF THE INVENTION

ENHANCED PROTEIN PRODUCTION IN HIGHER PLANTS BY N-TERMINAL FUSION OF A UBIQUITIN OR A CUCUMBER MOSAIC VIRUS COAT PROTEIN PEPTIDE

BACKGROUND OF THE INVENTION

Strategies for production of proteins in heterologous fusion form have been widely applied in biotechnology for many purposes, such as secretion of proteins from host cells (fused to signal peptides), easy detection or purification of protein products (fused to reporter enzymes for detection and to peptide tags for purification), searching for proteins with desired biological activities (e.g., in the phage display technique and the two-hybrid system). Enhanced expression of proteins of interest has also been achieved by N-terminal fusion of a small peptide to the target protein. Fusion of a ubiquitin gene together with a ubiquitin promoter to the 5'-end of a gene of interest is one of the systems which has been used to enhance protein expression. Ubiquitin exists in all eukaryotic cells and is the most highly conserved protein yet identified. It is abundant in cells and exhibits profound stability to heat and proteolytic degradation. Moreover, ubiquitin precursors, that is, polyubiquitin where ubiquitin monomers are linked up head to tail and ubiquitin extension proteins where a single ubiquitin is appended at its C-terminus to either of two small ribosomal proteins, undergo rapid processing by ubiquitin C-terminal hydrolases, which cleave C-terminal of the ubiquitin moieties and release the free ubiquitin monomer and the C-terminal extension proteins. All of these features have rendered ubiquitin as an excellent N-terminal fusion partner to augment target protein accumulation in genetic engineering.

The ubiquitin fusion approach was first developed by Butt et al. (1989), who showed that fusion of ubiquitin to yeast metallothionein or to the α subunit of the adenoylate cyclase-stimulatory GTP-binding protein increased the yield of these otherwise unstable or poorly expressed proteins from undetectable levels to 20% of the total cellular proteins in *E. coli*. Ecker et al. (1989) demonstrated that in yeast, ubiquitin fusion resulted in enhanced expression of three mammalian proteins by up to 200-fold and all these ubiquitin fusion proteins were correctly processed by yeast ubiquitin-specific endopeptidase to release authentic functional proteins. A similar yeast ubiquitin fusion expression system was reported by Sabin et al. (1989), in which ubiquitin/human γ-interferon and ubiquitin/α1-proteinase inhibitor were highly expressed and quantitatively cleaved to yield γ-IFN and α1-PI with authentic amino termini.

Since these early reports, a wealth of studies on ubiquitin fusion expression of various proteins in *E. coli* and yeast have been described (Baker et al., 1994; Barr et al., 1991; Coggan et al., 1995; Gali and Board, 1995; Gehring et al., 1995; Han et al., 1994; Kiefer et al., 1992; Lu et al., 1990; Lyttle et al., 1992; Mak et al., 1989; McDonnell et al., 1989; McDonnell et al., 1991; Pilon et al., 1996; Poletti et al., 1992; Rian et al., 1993; Tan and Board, 1996; Welch et al., 1995). Very often fusion to ubiquitin led to dramatic enhancement in yield of the fusion protein in bacteria, or of the cleaved product in yeast.

Enhanced expression of foreign proteins by ubiquitin fusion has also been observed in plants. In analysis of the promoter of the tobacco polyubiquitin gene, *Ubi.U4*, by driving transient expression of the GUS reporter in tobacco protoplasts, Genschik et al. (1994) found deletion of the intron sequence from the *Ubi.U4* fragment spanning from -263 to the end of the first ubiquitin-coding unit had no detectable influence on the GUS activity, but further deletion of the ubiquitin-coding sequence diminished the GUS activity by 55%.

None of these studies has shown the direct enhancing function of the ubiquitin fusion from a heterologous promoter. Garbarino and Belknap (1994) observed that fusion of the promoter plus ubiquitin-coding region of the potato ubiquitin extension protein gene *ubi 3* to the GUS reporter gene resulted in GUS activity 5- to 10-fold higher than the direct fusion of the *ubi 3* promoter to the GUS gene did in transgenic potato. Again, the synergistic effect of the *ubi 3* promoter and the ubiquitin-coding sequence on the enhanced GUS activity was not excluded. In another study with a potato polyubiquitin gene, *ubi 7*, the same group (Garbarino et al., 1995) demonstrated that in transgenic potato plants GUS expression level from the fusion construct containing the *ubi 7* promoter-5' untranslated sequence-intron-first ubiquitin coding unit was 10 times higher than that derived by only the *ubi 7* promoter with the 5' untranslated sequence. However, the effects of the intron and the ubiquitin protein fusion in increasing expression level of the GUS reporter were not clearly discriminated.

In addition to the above mentioned journal papers, a number of patents related to the ubiquitin fusion technology have been filed since 1989. They are shown in Table 1. The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference, and for convenience are respectively grouped in the appended List of References.

Table I

Patents related to the ubiquitin fusion technology

Title	Inventor	Patent No.	Filing Date	Host cells
Generating desired amino-terminal residue in protein	MIT	WO 8909829	10/19/1989	
Regulation metabolic stability of a protein	MIT	US 5093242	3/3/1992	mammal, yeast
Nucleic acid constructs, malaria polypeptides and vaccines	Chiron	WO 9208795	5/29/1992	yeast
Production of a protein with a predetermined amino-terminal amino acid residue	MIT	US 5196321	3/23/1993	E. coli
Yeast expression system for retinoid-X receptor	American Cyanamid	EP 608532	8/3/1994	yeast
Recombinant DNA vectors	Mascarenhas	WO 9423040	10/13/1994	E. coli
New heat-inducible N-degron protein and nucleic acid encoding it	Varshavsky, Dohmen, Johnston, Wu	WO 9521269	8/10/1995	
Fusion proteins containing the N-or C-terminal of ubiquitin	Varshavsky, Johnston	WO 9529195	11/2/1995	
New fusion protein of ubiquitin plant and lytic peptide	Carbarino, Jaynes, Belknap	WO 9603519	2/8/1996	plant
Production of tissue factor pathway-inhibitor in yeast cells	Innis, Creasey	WO 9604377	2/15/1996	yeast
Stable recombinant ubiquitin-lytic peptide fusion protein	J. Jaynes	WO 9603522	2/8/1996	plant
Fusion protein encoded by a gene construct	Bachmair, Finley, Varshavsky	US 5496721	5/3/1990	mammal, yeast

SUMMARY OF THE INVENTION

In accordance with the present invention a method for enhancing expression of proteins in plants or plant cells is achieved by the fusion of a ubiquitin monomer coding sequence to the 5' end of the coding sequence of the proteins. Expression of the ubiquitin fusion proteins is driven by a promoter other than promoters from polyubiquitin protein genes or ubiquitin extension protein genes. Thus enhancement of expression level of the proteins is due to the 5' terminal addition of the ubiquitin monomer coding sequence. The ubiquitin fusion proteins are cleaved at the carboxy-terminal glycine 76 residue of the ubiquitin, presumably by plant ubiquitin specific proteases, to produce proteins with desired biological properties. A second aspect of this invention is that the N-terminal peptide of 14 amino acid residues of cucumber mosaic virus coat protein (NP14) can be used as an N-terminal fusion partner to increase the expression level of target proteins in plants. The N-terminal fusion approaches described in this invention allow higher yield production of proteins in plants, either in the authentic forms in the ubiquitin fusion system or as the fusion protein in the NP14 fusion system.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleotide sequence and deduced amino acid sequence of tobacco *ubi.NC89*. The nucleotide sequence is listed as SEQ ID NO:1 and the amino acid sequence is SEQ ID NO:2 in the Sequence Listing. The primers used in PCR are underlined and the mended 37-mer oligonucleotide is double-underlined.

Figure 2 shows the synthetic DNA coding for the 14 N-terminal amino acids of CMV CP (NP14). The nucleotide sequence is SEQ ID NO:3 and the amino acid sequence is SEQ ID NO:4.

Figure 3 illustrates the construction of the ubiquitin-GUS fusion protein expression vector pUG. The nucleotide sequence shown for pSKUBC1 is SEQ ID NO:5, the sequence shown for pBI221 is SEQ ID NO:6, and the sequence shown for pUG is SEQ ID NO:7.

Figure 4 illustrates the construction of the NP14-GUS fusion protein expression vector pCG. The nucleotide sequence shown for pUCG2 is SEQ ID NO:8.

Figure 5 illustrates the construction of the ubiquitin-luciferase fusion protein expression vector pUL. The arrow marked in the recognition sequence of Stu I in pBIubi indicates the end of the ubiquitin coding region and the cleavage site of the ubiquitin fusion protein. The upper

nucleotide sequence shown for pBIubi is SEQ ID NO:9, the lower nucleotide sequence shown for pBIubi is SEQ ID NO:10, and the nucleotide sequence shown for pUL is SEQ ID NO:11.

Figure 6 illustrates the construction of the NP14-luciferase fusion protein expression vector. The nucleotide sequence shown for pCL is SEQ ID NO:12.

Figure 7 illustrates the construction of ubiquitin-GUS fusion/LUC dual report binary vector pUGL121.

Figure 8 illustrates the construction of the NP14-GUS fusion/LUC dual reporter binary vector pCGL121.

Figure 9 illustrates the construction of the GUS/LUC dual reporter binary vector pBIL121.

Figure 10 illustrates the ubiquitin fusion cloning vector pBIubi. The upper nucleotide sequence is SEQ ID NO:13 and the lower nucleotide sequence is SEQ ID NO:14.

Figure 11 illustrates the NP14 fusion cloning vector pBINP14.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to methods and constructs for enhancing protein production in plants. The methods comprise fusing an expression-enhancing nucleic acid at the 5' terminus of the gene for which enhanced expression is desired. In one aspect of the invention, a ubiquitin gene is inserted in front of the gene encoding the desired protein such that a fusion protein is produced wherein ubiquitin is directly fused to the amino terminus of the desired protein. Enzymes such as C-terminal hydrolases, will cleave at the C-terminus of the ubiquitin in the fusion protein thereby releasing the desired protein in its natural form as well as forming free ubiquitin. The presence of the ubiquitin gene in the resulting fusion protein results in enhanced expression of the gene thereby yielding a greater amount of the desired protein product than occurs in the absence of the ubiquitin gene. It is necessary to use only the coding portion of the ubiquitin gene. The ubiquitin promoter is unnecessary, and the ubiquitin gene fusion can be under the control of a heterologous promoter.

In a second aspect of the invention, enhanced protein production is seen when a nucleic acid encoding 14 amino acids of cucumber mosaic virus coat protein is placed in front of the gene encoding a desired protein such that a fusion protein is produced wherein the fusion protein

includes the 14 amino acids of the cucumber mosaic virus coat protein at the amino terminus of the fusion protein.

The aspects of the invention are set out in the following Examples which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized. Techniques such as transfection of protoplasts, preparation of transgenic tobacco plants, fluorometric GUS assays and luciferase assays are well known to those of skill in the art and are not described in detail herein.

EXAMPLE 1

DNA Sequences Coding for the Tobacco Ubiquitin and the N-terminal Peptide of CMV Coat Protein

The coding sequence of the ubiquitin monomer contains 228 base pairs. The 5' part of 191 base pairs was obtained by polymerase chain reaction (PCR) amplification on the total DNA of *Nicotiana tobacum* var. NC89 and the remaining 37 base pairs were prepared as a synthetic oligonucleotide. An SphI site encompassing the initiation codon ATG and an NcoI site following the last codon GGC were created to facilitate cloning. The tobacco ubiquitin coding sequence was then cloned into pGEM-5ZF and sequenced. Figure 1 shows the DNA sequence and the deduced amino acid sequence of the tobacco ubiquitin. The 76-amino acid sequence is identical to that derived from a tobacco polyubiquitin gene *ubi.U4* (Genschik et al., 1994). However, the nucleotide sequence of the region amplified from the tobacco DNA is different from the corresponding regions of all ubiquitin monomers found in *ubi.U4*. We have named this tobacco ubiquitin coding sequence as *ubi.NC89*.

The cucumber mosaic virus coat protein (CMV CP) is encoded by the viral subgenomic RNA 4 and comprises 218 amino acid residues. The CP gene of the strain CMV-SD was cloned by RT-PCR (Guo et al., 1993) and the cDNA sequence encoding the 14 N-terminal amino acids (NP14) was either cut out of the CP gene by NcoI/AccI digestion or chemically synthesized. In the synthesized version of the NP14 coding sequence (Figure 2), overhanging adapters for BamHI and SstI sites were attached to the 5'- and 3'-ends, respectively, for easy cloning.

EXAMPLE 2

Translational Fusion Constructs for Transient Expression Assays

A. Ubiquitin-GUS fusion construct pUG

The *ubi.NC89* sequence was taken from the plasmid pSKUBC1 as an XbaI-NcoI (filled-in) fragment and inserted into the XbaI-BamHI (filled-in) site upstream of the GUS gene in pBI221 to construct pUG as shown in Figure 3.

B. NP14-GUS fusion construct pCG

Plasmid pUCG2 is a derivative of pBI221, in which the *ubi.NC89* sequence and the NP14 sequence, linked as a read-through ORF, was inserted into the XbaI-SmaI sites in front of the GUS gene. The ubiquitin moiety was removed from pUCG2 by XbaI-SacII digestion and pCG was formed by recircularizing. Figure 4 illustrates these steps clearly.

C. Ubiquitin-LUC fusion construct pUL

An NcoI (filled-in)-SstI fragment containing the firefly luciferase (LUC) gene was inserted into the ubiquitin fusion vector pBIubi (see Figure 10) downstream of *ubi.NC89* via the StuI-SstI sites in the polylinker region, resulting in pUL as shown in Figure 5.

D. NP14-LUC fusion construct pCL

The NcoI (filled-in)-SstI fragment containing the LUC gene was inserted into the NP14 fusion vector pBINP14 (see Figure 11) downstream of the NP14 coding sequence via AccI (or SalI which is the equivalent site here) (filled-in)-SstI sites, resulting in pCL as shown in Figure 6.

EXAMPLE 3

GUS/LUC Dual Reporter Constructs for Stable Transformation

To examine the enhancing effects of the N-terminal addition of the ubiquitin or CMV CP NP14 on GUS expression in stably transformed plants, a series of GUS/LUC (test/reference) dual reporter constructs were made. Essentially they are based on the fusion constructs used in transient expression assays, namely, pUG and pCG. The chimeric GUS expression cassettes were moved into the plant transformation intermediate plasmid pBI121, resulting in pUG121 and

pCG121, respectively. The expression cassette of the reference reporter LUC, which was constructed by replacing the GUS gene in pBI221 with the LUC gene, was pre-made as a HindIII fragment (HindIII-35S/LUC/NOS-HindIII) and then inserted into the unique HindIII site of pUG121, pCG121 and pBI121, respectively. The resulting GUS/LUC dual reporter constructs, pUGL121, pCGL121 and pBIL121 are shown in Figures 7, 8 and 9, respectively.

EXAMPLE 4

Ubiquitin fusion enhances the expression of GUS and LUC in tobacco protoplasts

The ubiquitin-GUS fusion construct pUG or the control plasmid pBI221 was introduced into tobacco protoplasts derived from tobacco BY-2 suspension cells, together with a reference plasmid FFO which contained LUC gene driven by the 35S promoter. GUS activities were determined and normalized by luciferase activities. In four independent transfection experiments, the normalized GUS activities (ΔGUS) from pUG were considerably higher than those from pBI221. The averaged increase fold due to the ubiquitin fusion is 6.0 (Table 2). When using LUC as a reporter and GUS as an internal standard as expressed from pBI221, the normalized LUC activities from pUL were 1.37 to 3.11 fold higher than those from the control plasmid p35SLUC (35S-LUC-NOS) in three independent transfection experiments, with the average increase fold about 2 (Table 3).

EXAMPLE 5

CMV CP NP14 Is a More Efficient Fusion Partner than Ubiquitin

The enhancing effects of the NP14 fusion on GUS and LUC expression in tobacco protoplasts were examined in experiments parallel to the above mentioned ubiquitin fusion study. The NP 14-GUS fusion construct pCG produced an average 11-fold higher GUS activity than did pBI221. These results are shown in Table 2. Fusion of NP14 to LUC increased the LUC activity by 2.87 times, calculated by comparing the normalized LUC activity of pCL to that of p35SLUC. These results are shown in Table 3. It is apparent that NP14 is a more efficient fusion partner than ubiquitin in augmenting GUS and LUC expression in tobacco cells.

Table 2

Normalized GUS activities and enhancing fold of the N-terminal fusion constructs

plasmid	pBI221	pUG		pCG	
activities	GUS	ΔGUS	E	ΔGUS	E
1	293.3	3760.0	12.8	5743.0	19.6
2	206.7	584.3	2.8	940.8	4.6
3	856.7	3733.8	4.4	6708.0	7.8
4	100.0	408.8	4.1	1247.0	12.5
average E value		6.0±2.2		11.1±3.2	

Notes: 1. The normalized GUS activity ΔGUS is calculated by the formula

$$\Delta GUS_n = \frac{GUS_n \times LUC_{221}}{LUC_n}$$

where n represents a particular GUS fusion construct, 221 represents pBI221.

2. The enhancing fold E is calculated as $\frac{\Delta GUS_n}{GUS_{221}}$

Table 3

Normalized LUC activities and enhancing fold of the N-terminal fusion constructs

Plasmid		p35S LUC		pUL			pCL		
activities		ΔLUC	average ΔLUC	ΔLUC	average ΔLUC	E	ΔLUC	average ΔLUC	E
1	1	252	290	274	396	1.37	457	491	1.70
	2	329		518			529		
2	1	169	169	556	526	3.11	701	794	4.70
	2	ND		496			886		
3	1	64	112	141	164	1.46	270	246	2.20
	2	160		181			254		
	3	ND		170			214		
Mean±SE				1.98±0.56			2.87±0.92		

Notes: 1. The normalized LUC activity ΔLUC is calculated by the formula

$$\Delta LUC_n = \frac{LUC_n \times GUSp35SLUC}{GUS_n}$$

where n represents a particular LUC fusion construct.

2. The enhancing fold E is calculated as

$$\frac{\Delta LUC_n}{LUCp35SLUC}.$$

EXAMPLE 6

Ubiquitin- and NP14-fusion Enhance GUS Expression in Transgenic Plants

To examine the enhancing effects of the ubiquitin fusion and the NP14 fusion on GUS expression in stably transformed plants, three GUS/LUC (test/reference) dual reporter constructs were made based on the binary vector pBI121. pUGL121, pCGL121 and pBIL121 contained expression cassettes ubiquitin-GUS, NP14-GUS and GUS only (control), respectively, and the reference LUC expression cassette was integrated in each plasmid (Figures 7-9). Tobacco plants transformed with each of the three constructs were prepared and analyzed for GUS and LUC activities. Each plant was analyzed twice in two independent experiments and only those plants displaying reasonable consistency of the relative GUS activities (GUS/LUC) in two experiments were included for comparison. As shown in Table 4, although variations in the relative GUS activities existed among different transformants from the same constructs, the average GUS expression level of 5 qualified plants containing the 35S-ubiquitin/GUS fusion construct was 4 times higher than that derived from 6 plants containing the 35S-GUS construct, confirming the enhancing effect of the ubiquitin fusion on GUS expression as previously observed in tobacco protoplasts. Again, the NP14 fusion displayed a higher enhancing effect on GUS expression than did the ubiquitin fusion. The average relative GUS activity of 14 pCGL plants was about 7 fold that derived from the pBIL121 construct.

EXAMPLE 7

Ubiquitin fusion and NP14 fusion cloning vectors

pBIubi (Figure 10) and pBINP14 (Figure 11) are two fusion protein expression vectors allowing for insertion of target genes downstream of the *ubi.NC89* and the CMV CP NP14 coding sequence, respectively. Both vectors are derivatives of pBI221, with the GUS gene being replaced by the *ubi.NC89* or NP14 coding sequence. In pBIubi, a polylinker sequence was attached to the 3' end of the *ubi.NC89* sequence and the penultimate codon of the *ubi.NC89* was changed from GGT to GGA for creating a StuI site in the polylinker region. In pBINP14, two cloning sites, SalI (here equivalent to an AccI site) and SstI, are available for cloning the target genes downstream from the NP14 sequence (the last 5 base pairs of the NP14 sequence form part of the SalI recognition sequence). In order to use AccI instead of SalI for cleaving pBINP14, the AccI site at -393 of the CaMV 35S promoter was eliminated.

Table 4

Effects of ubiquitin- and NP14-fusion on GUS expression in transgenic tobacco plants

Plant lines	Relative GUS activities: GUS/LUC (pmol $MU \cdot min^{-1}/cpm \times 10^{-3}$)								
	pUGL121			pCGL121			pBIL121		
	exp. 1	exp. 2	average	exp. 1	exp. 2	average	exp. 1	exp. 2	average
1	12.9	15.3	14.1	2.4	3.4	2.9	1.4	2.6	2
2	13	43	28	4.5	6.8	5.65	5.2	2.4	3.8
3	0.7	0.5	0.6	63.2	9.5	36.35	4.2	0.6	2.4
4	0.3	0.4	0.35	26.9	8.3	17.6	2.5	5.4	3.95
5	4.8	0.8	2.8	17.8	22.2	20	0.4	0.38	0.39
6				2.1	5	3.55	0.5	0.82	0.66
7				4.6	5.8	5.2			
8				58.7	20.2	39.45			
9				15.6	3.6	9.6			
10				17.2	4.4	10.8			
11				3	1.4	2.2			
12				17.9	24.2	21.05			
13				20.7	19.4	20.05			
14				13.7	25.3	19.5			
Mean ±SE	9.17±5.34			15.28±3.18			2.2±0.61		

While the invention has been disclosed by reference to the details of preferred embodiments of the invention, it is to be understood that the disclosure is intended in an illustrative rather than in a limiting sense, as it is contemplated that modifications will readily occur to those skilled in the art, within the spirit of the invention and the scope of the appended claims.

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50 55 60

Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly
65 70 75

<210> 3
<211> 53
<212> DNA
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<220>
<221> CDS
<222> (6)..(47)

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gatcc atg gac aaa tct gaa tca acc agt gct ggt cgt aac cgt cga 47
Met Asp Lys Ser Glu Ser Thr Ser Ala Gly Arg Asn Arg Arg
1 5 10

cgagct 53

<210> 4
<211> 14
<212> PRT
<213> cucumber mosaic virus

<400> 4
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1 5 10

<210> 5
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<212> DNA
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<220>
<221> misc_feature
<222> ()..)
<223> Joining region of fusion of two genes.

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<210> 6
<211> 33
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<213> Plasmid pBI221

<220>
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<211> 18
<212> DNA
<213> Plasmid pUG

<220>
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<223> Joining region of fusion of genes.

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<210> 8
<211> 18
<212> DNA
<213> Plasmid pUCG2

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<210> 9
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<213> Plasmid pBIubi

<220>
<221> misc_feature
<222> (1)..(29)
<223> Joining region between promoter and fused gene.

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<210> 10
<211> 35
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<220>
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polylinker sequence.

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<210> 11
<211> 12
<212> DNA
<213> Plasmid pUL

<220>
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<222> (1)..(12)
<223> Joining region between fusion of genes.

<400> 11
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<210> 12
<211> 12
<212> DNA
<213> Plasmid pCL

<220>
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<222> (1)..(12)
<223> Joining region between fusion of genes.

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cgtcgcatgg aa 12

<210> 13
<211> 29
<212> DNA
<213> Plasmid pBIubi

<220>
<221> misc_feature
<222> (1)..(29)
<223> Joining region of fusion of promoter and gene.

<400> 13
tctagaacta gtggatccct ggcatgcag 29

<210> 14
<211> 35
<212> DNA
<213> Plasmid pBIubi

<220>
<221> misc_feature
<222> (1)..(35)
<223> Joining region with multicloning sequence between fusion of gene and terminator.

<400> 14
ggaggcctgt cgactcgagc ccgggtaccg agctc 35

WHAT IS CLAIMED IS:

1. A method for enhancing production of a desired protein in a plant cell or a plant which comprises inserting a first nucleic acid upstream of a second nucleic acid to form a fused nucleic acid wherein said first nucleic acid encodes a ubiquitin monomer and wherein said second nucleic acid encodes said desired protein and further wherein said fused nucleic acid encodes a fusion protein and further wherein expression of said fusion protein is not under the control of a ubiquitin promoter.

2. The method of claim 1 wherein said ubiquitin monomer consists of SEQ ID NO:2.

3. The method of claim 1 wherein the carboxy terminus of said ubiquitin forms a peptide linkage with the amino terminus of said desired protein.

4. The method of claim 1 wherein said first nucleic acid comprises bases 3-230 of SEQ ID NO:1.

5. The method of claim 1 wherein said fused nucleic acid is under the control of a 35S promoter.

6. A method for enhancing production of a desired protein as part of a fusion protein in a plant cell or a plant which comprises inserting a first nucleic acid upstream of a second nucleic acid to form a fused nucleic acid wherein said first nucleic acid encodes a protein of SEQ ID NO:4 and wherein said second nucleic acid encodes said desired protein and further wherein said fused nucleic acid encodes said fusion protein.

7. The method of claim 6 wherein the carboxy terminus of said protein of SEQ ID NO:4 forms a peptide linkage with the amino terminus of said desired protein.

8. The method of claim 6 wherein said first nucleic acid comprises bases 6-47 of SEQ ID NO:3.

9. The method of claim 6 wherein said fused nucleic acid is under the control of a 35S promoter.

10. A nucleic acid vector capable of transforming a plant cell wherein said vector comprises nucleic acid which encodes a fusion protein wherein said fusion protein comprises a ubiquitin monomer linked to a protein of interest and further wherein expression of said fusion protein is not under the control of a ubiquitin promoter.

11. The vector of claim 10 wherein said ubiquitin consists of SEQ ID NO:2.

12. The vector of claim 10 wherein said ubiquitin is linked in a peptide linkage at its carboxy terminus to the amino terminus of said protein of interest.

13. The vector of claim 10 wherein said nucleic acid is under the control of a 35S promoter.

14. The vector of claim 10 wherein said vector comprises bases 3-230 of SEQ ID NO:1.

15. A nucleic acid vector capable of transforming a plant cell wherein said vector comprises a nucleic acid which encodes a fusion protein wherein said fusion protein comprises a protein of SEQ ID NO:4 linked to a protein of interest.

16. The vector of claim 15 wherein said protein of SEQ ID NO:4 is linked in a peptide linkage at its carboxy terminus to the amino terminus of said protein of interest.

17. The vector of claim 15 wherein said nucleic acid is under the control of a 35S promoter.

18. The vector of claim 15 wherein said vector comprises bases 6-47 of SEQ ID NO:3.

19. A plant cell or a plant comprising the vector of claim 10.

20. A plant cell or a plant comprising the vector of claim 15.

21. A nucleic acid comprising SEQ ID NO:1.

22. A nucleic acid consisting of SEQ ID NO:1.

23. A nucleic acid comprising SEQ ID NO:3.

24. A nucleic acid consisting of SEQ ID NO:3.

25. A protein comprising SEQ ID NO:2.

26. A protein consisting of SEQ ID NO:2.

27. A protein consisting of SEQ ID NO:4.

28. A fusion protein wherein said fusion protein comprises a ubiquitin monomer at the amino terminus of said fusion protein and wherein said fusion protein comprises a second protein at its carboxy terminus.

29. The fusion protein of claim 28 wherein said ubiquitin monomer consists of SEQ ID NO:2.

30. The fusion protein of claim 28 wherein the carboxy terminus of said ubiquitin monomer forms a peptide linkage with the amino terminus of said second protein.

31. A fusion protein wherein said fusion protein comprises a protein of SEQ ID NO:4 at the amino terminus of said fusion protein and wherein said fusion protein comprises a second protein at its carboxy terminus.

32. The fusion protein of claim 31 wherein the carboxy terminus of said protein of SEQ ID NO:4 forms a peptide linkage with the amino terminus of said second protein.

```
BamHI
    1   M    D   K    S   E    S   T   S
 GATCCATG GAC AAA TCT GAA TCA ACC AGT
      TAC CTG TTT AGA CTT AGT TGG TCA

 A    G   R    N   R    R   14
GCT GGT CGT AAC CGT CGA CGAGCT
CGA CCA GCA TTG GCA GCT GC
                 AccI    SstI
```

FIG. 2

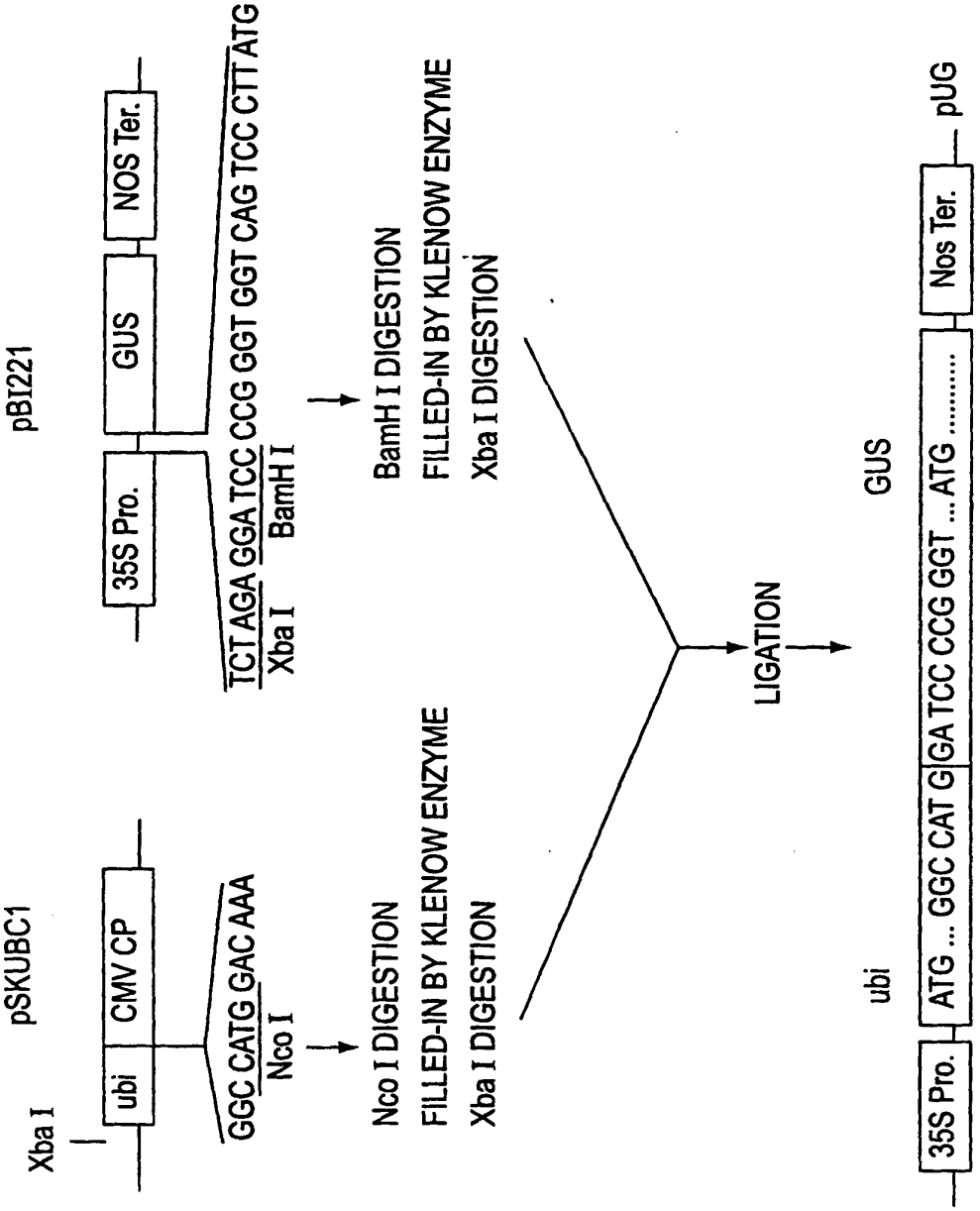

pSKUBC1
Xba I
ubi
CMV CP
GGC CATG GAC AAA
Nco I
Nco I DIGESTION
FILLED-IN BY KLENOW ENZYME
Xba I DIGESTION
pBI221
35S Pro.
GUS
NOS Ter.
TCT AGA GGA TCC CCG GGT GGT CAG TCC CTT ATG
Xba I BamH I
BamH I DIGESTION
FILLED-IN BY KLENOW ENZYME
Xba I DIGESTION
LIGATION
ubi
35S Pro.
ATG ... GGC CAT G GA TCC CCG GGT ... ATG .....
GUS
Nos Ter.
pUG

FIG. 3

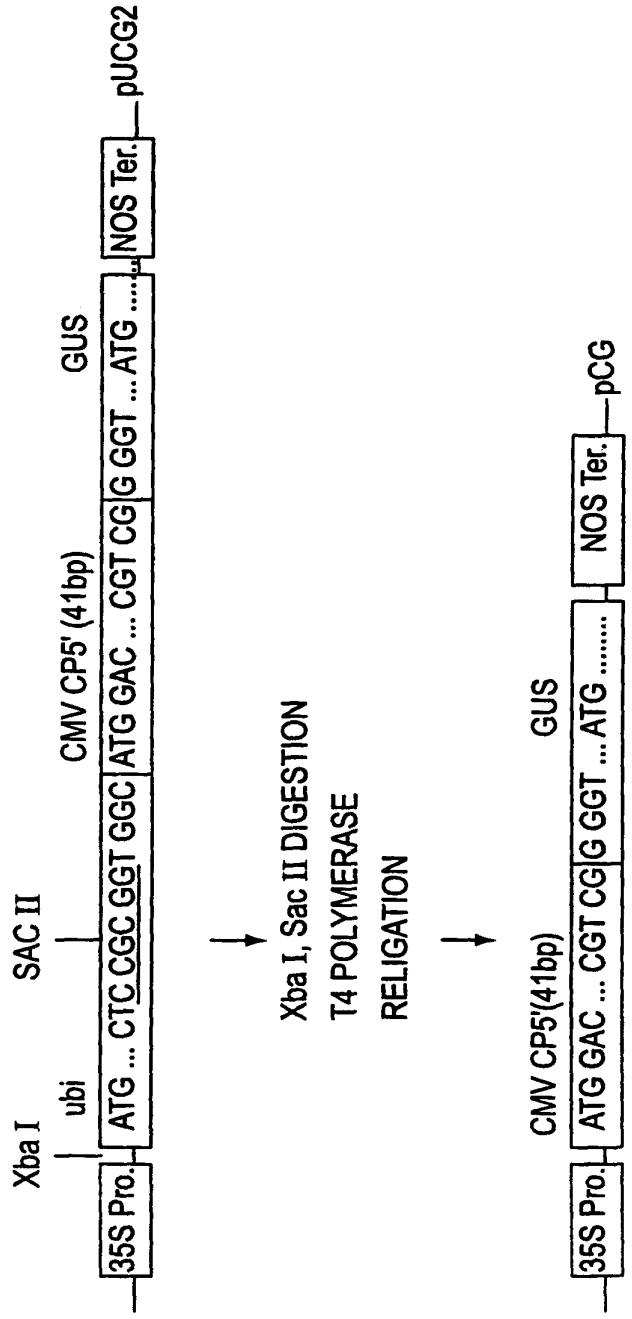

Xba I
ubi
SAC II
CMV CP5' (41bp)
GUS
pUCG2
35S Pro.
ATG ... CTC CGC GGT GGC
ATG GAC ... CGT CG
G GGT ...ATG .....
NOS Ter.
Xba I, Sac II DIGESTION
T4 POLYMERASE
RELIGATION
CMV CP5'(41bp)
GUS
pCG
35S Pro.
ATG GAC ... CGT CG
G GGT ...ATG .....
NOS Ter.

FIG. 4

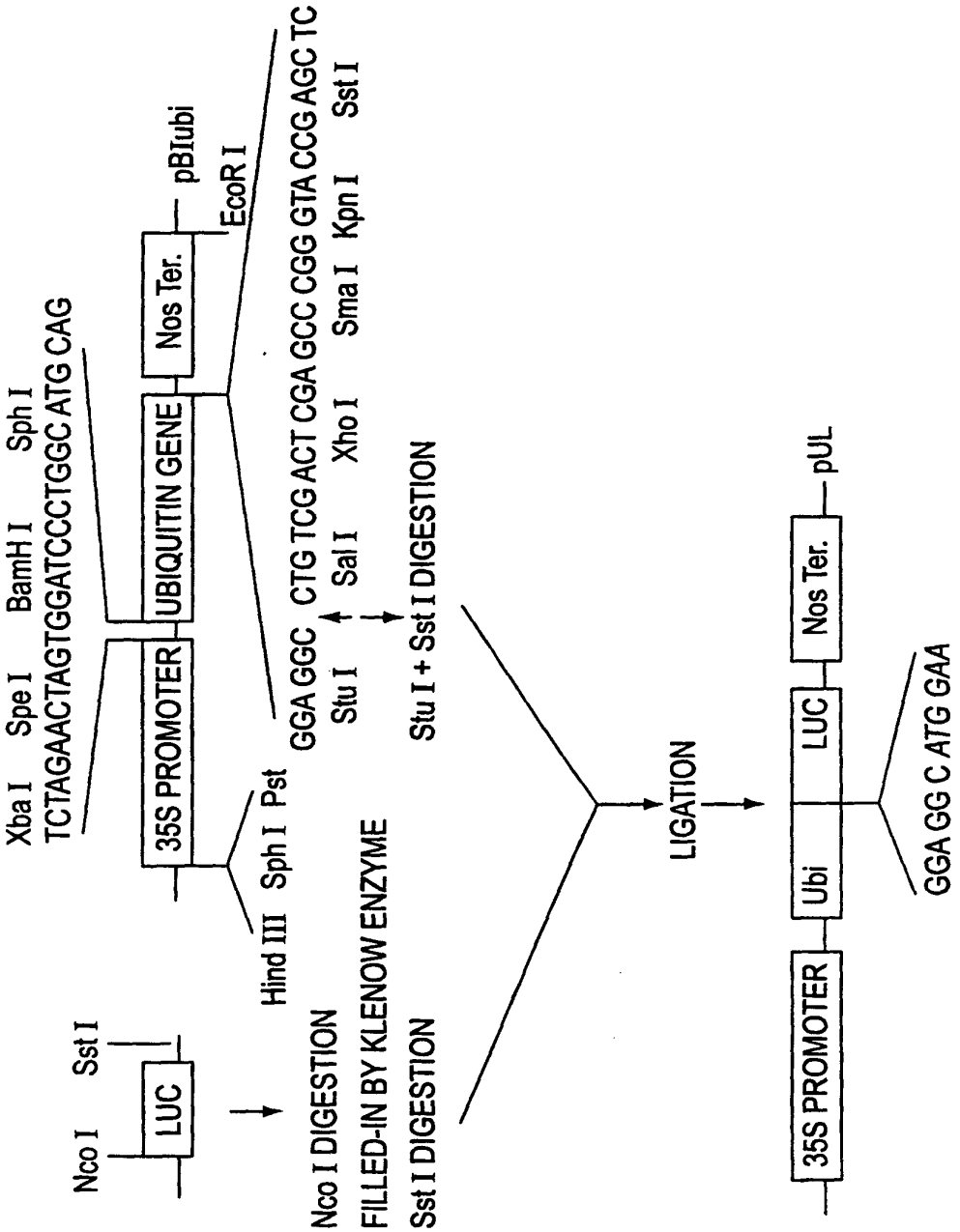
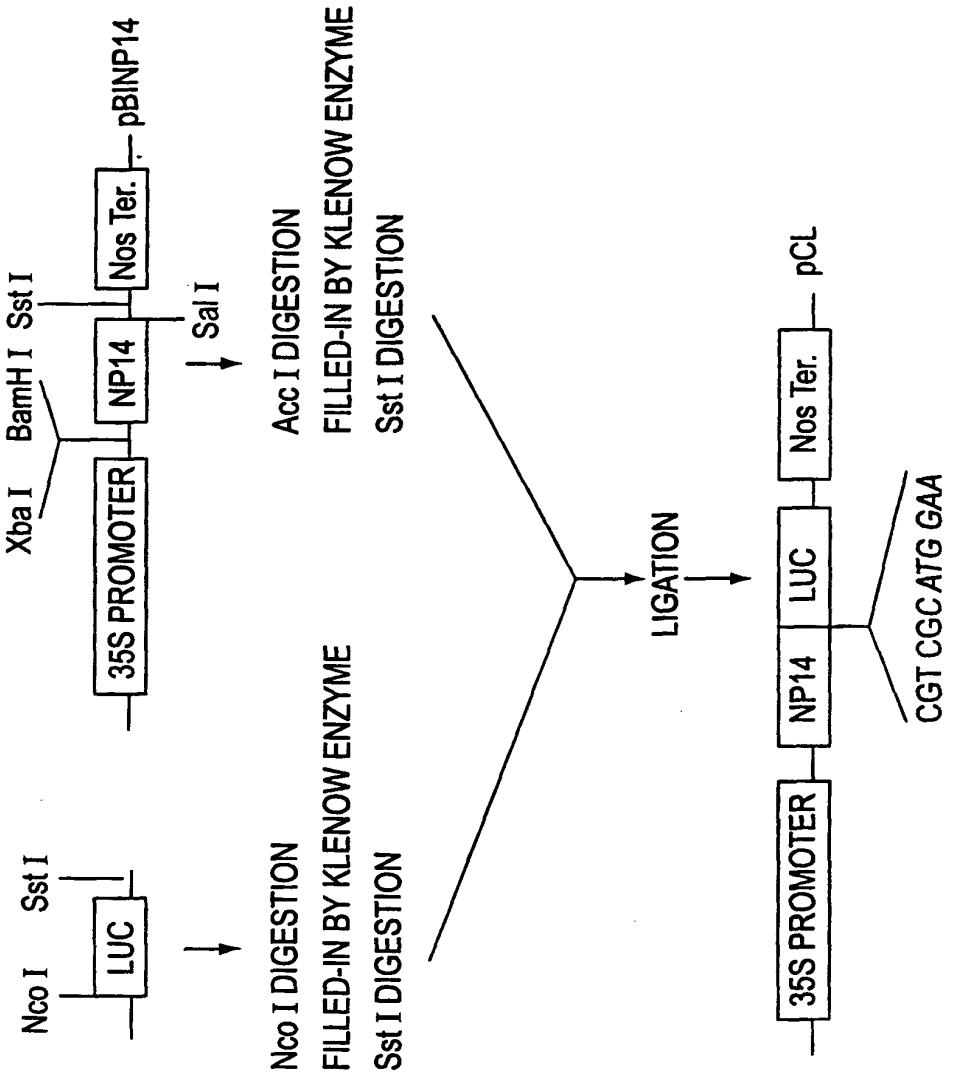

Xba I Spe I BamH I Sph I
TCTAGAACTAGTGGATCCCCTGGC ATG CAG
35S PROMOTER
UBIQUITIN GENE
Nos Ter.
pBIubi
EcoR I
GGA GGC CTG TCG ACT CGA GCC CGG GTA CCG AGC TC
Stu I Sal I Xho I Sma I Kpn I Sst I
Hind III Sph I Pst
Nco I Sst I
LUC
Nco I DIGESTION
FILLED-IN BY KLENOW ENZYME
Sst I DIGESTION
Stu I + Sst I DIGESTION
LIGATION
35S PROMOTER
Ubi
LUC
Nos Ter.
pUL
GGA GG C ATG GAA

FIG. 5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BamH I
Sst I
35S PROMOTER
NP14
Nos Ter.
pBINP14
Sal I
Acc I DIGESTION
FILLED-IN BY KLENOW ENZYME
Sst I DIGESTION
Nco I
Sst I
LUC
Nco I DIGESTION
FILLED-IN BY KLENOW ENZYME
Sst I DIGESTION
LIGATION
35S PROMOTER
NP14
LUC
Nos Ter.
pCL
CGT CGC ATG GAA

FIG. 6

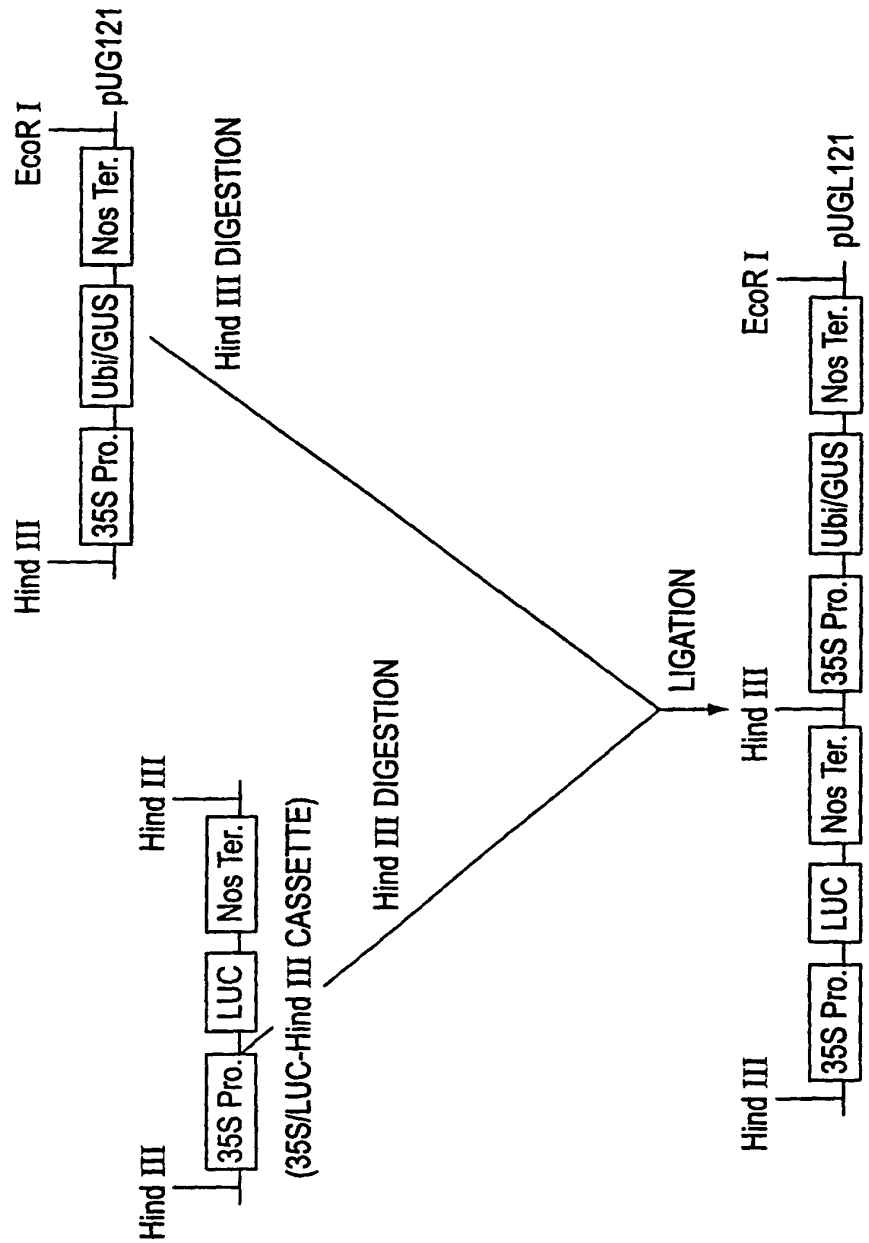
EcoR I
pUG121
Nos Ter.
Ubi/GUS
35S Pro.
Hind III
Hind III DIGESTION
Hind III
Nos Ter.
LUC
35S Pro.
Hind III
(35S/LUC-Hind III CASSETTE)
Hind III DIGESTION
LIGATION
EcoR I
pUGL121
Nos Ter.
Ubi/GUS
35S Pro.
Hind III
Nos Ter.
LUC
35S Pro.
Hind III

FIG. 7

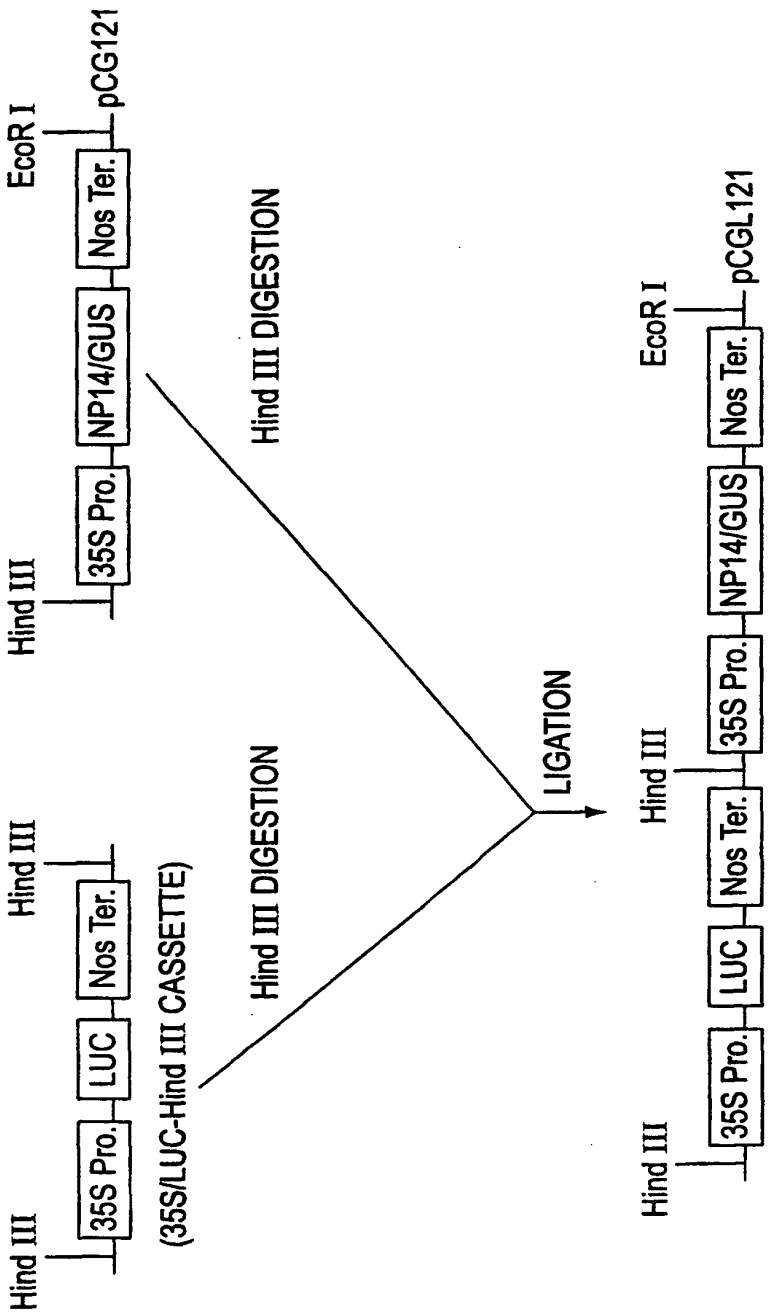
EcoR I
pCG121
Nos Ter.
NP14/GUS
35S Pro.
Hind III
Hind III DIGESTION
Hind III
Nos Ter.
LUC
35S Pro.
Hind III
(35S/LUC-Hind III CASSETTE)
Hind III DIGESTION
LIGATION
EcoR I
pCGL121
Nos Ter.
NP14/GUS
35S Pro.
Hind III
Nos Ter.
LUC
35S Pro.
Hind III

FIG. 8

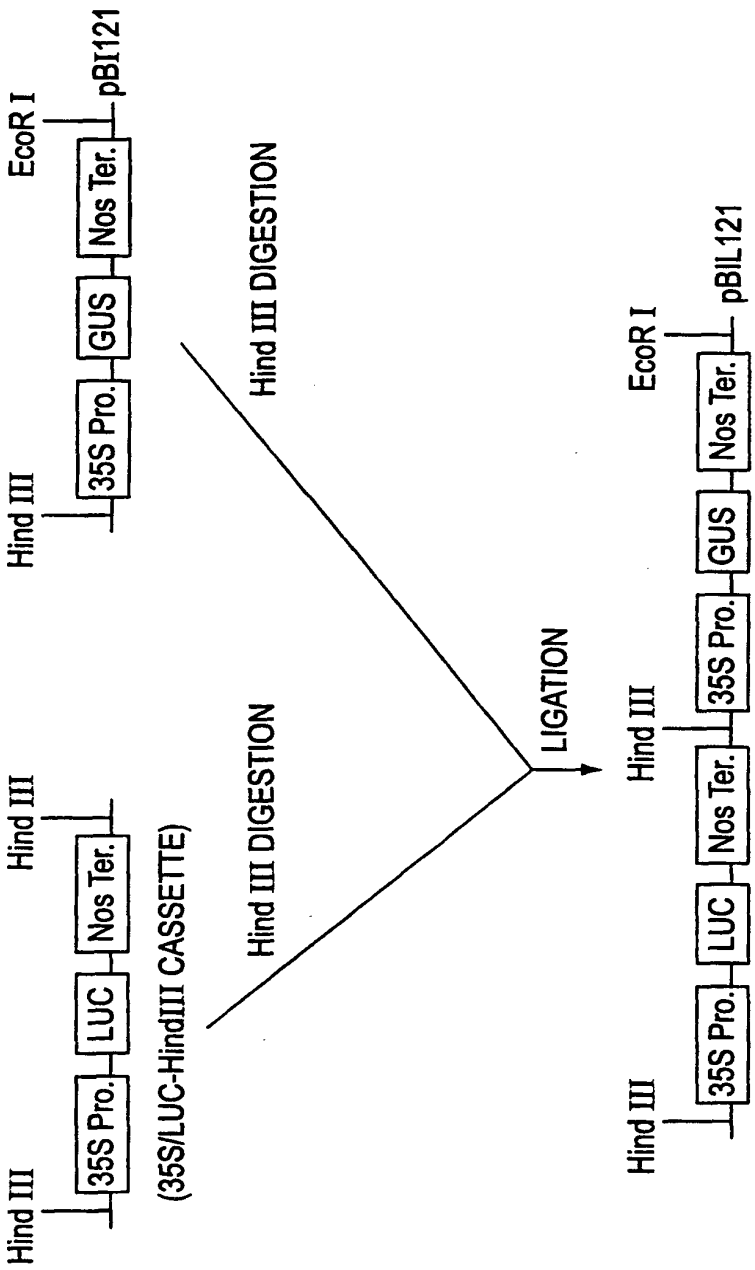
EcoR I
pBI121
Hind III
35S Pro.
GUS
Nos Ter.
Hind III DIGESTION
Hind III
35S Pro.
LUC
Nos Ter.
(35S/LUC-HindIII CASSETTE)
Hind III DIGESTION
LIGATION
EcoR I
pBIL121
Hind III
35S Pro.
GUS
Nos Ter.
Hind III
35S Pro.
LUC
Nos Ter.
Hind III
35S Pro.

FIG. 9

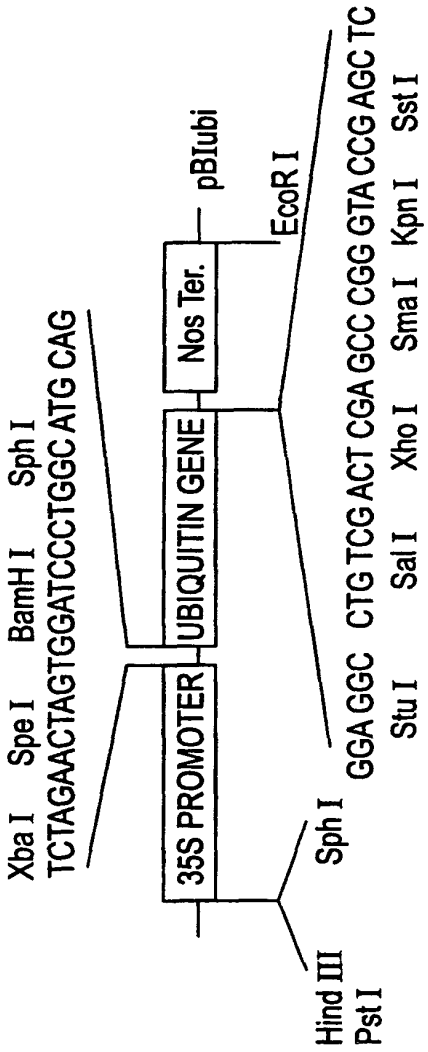
Xba I Spe I BamH I Sph I
TCTAGAACTAGTGGATCCCCTGGC ATG CAG
35S PROMOTER
UBIQUITIN GENE
Nos Ter.
pBIubi
EcoR I
GGA GGC CTG TCG ACT CGA GCC CGG GTA CCG AGC TC
Stu I Sal I Xho I Sma I Kpn I Sst I
Sph I
Hind III
Pst I

FIG. 10

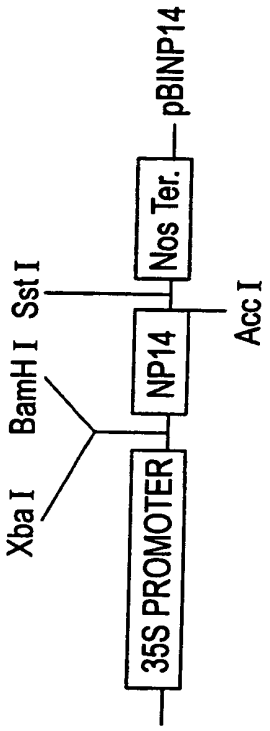

FIG. 11